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14. ABSTRACT

The goal of our research is to identify combinations of currently approved drugs that would be effective for treating MPNSTs. To pursue this goal, first we developed in vitro models by growing new cell lines from surgical specimens. In addition, we obtained established MPNST cells from outside collaborators. Second, we tested the cell lines for their ability to form tumor xenografts in Athymic nude mice. Third, we developed NF1 isogenic cells, where the only difference is the NF1 expression. These paired MPNST cells are currently being used in a large screen of FDA approved drugs. The resources attained during the first phase of this project are going to be very valuable for the subsequent drug screen step.

15. SUBJECT TERMS

MPNST, Neurofibroma, Neurofibromatosis Type 1, NF1, isogenic cells, drug screen

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INTRODUCTION

About one half of Malignant Peripheral Nerve Sheath Tumors (MPNSTs) arise in Neurofibromatosis Type 1 patients (NF1). Currently, these tumors present very poor clinical prognosis, and despite efforts to find better chemotherapy, there has been little impact on overall patient survival. The extent of surgical resection is the best prognostic factor for these tumors. Our goal is to identify new combination of receptor tyrosine kinase inhibitors that are effective in cell culture and animal models of MPNSTs. We expect finding effective drug combinations that can be used to treat MPNST patients.

BODY

Here we present the major research accomplishments for the first year of the development of this project. We successfully developed new MPNST cells derived from surgical specimens provide by the Johns Hopkins Hospital. We also obtained established MPNSTs cells from outside collaborators. This set of cells is essential for the *in vitro* drug testing and further *in vivo* characterization of drug efficacy. In addition, in order to accomplish the research described on Aim 3 of the project, we tested all cell lines for their ability to form xenografts in Athymic nude mice. Moreover, we developed NF1 isogenic cells. These cells differ only on the expression of the NF1 gene, which is the major genetic alteration in these tumors. We further tested the efficacy of a small number of drugs in reducing cell proliferation of these NF1 isogenic cells. We anticipate being able to screen a large library of FDA approved drugs using these reagents developed on the first year of this research.

Task1- Establishment of three new MPNST cells

Surgically resected MPNST tumors were dissociated and cells were cultured in neural stem cell media made of Neurobasal medium (Invitrogen) containing the mitogenic factors EGF and FGF. We also cultured these cells in standard serum containing medium with 10% of Fetal Bovine Serum. To date, cells cultured in neural stem cells medium grew for a few passages and rapidly senesced. This might be attributed to the specific genetic background of tumors, as cultured primary cells regularly differ in cell proliferation rates and therefore in the ability to form new cell lines. Up to the present time, we have successfully grown three new MPNST cells in serum containing medium and currently have cultured these cells for several passages. On Figure 1 the *in vitro* characteristic growth pattern of these cells is shown. Meanwhile, we are continually attempting to develop MPNST neurospheres, as new surgical specimens are made available. We also obtained established MPNST cells kindly provided by outside collaborators. A complete list of MPNST cells is shown on Table 1.

Task 2- In vivo tumor growth and characterization of MPNST cells

In order to develop a consistent *in vivo* MPNST model we implanted all available MPNST cells in Athymic nude mice. MPNST cells growing in culture were trypsinized, counted and resuspended in Matrigel (BD Biosciences). $5x10^6$ cells resuspended in 100µl of Matrigel were implanted subcutaneously in the middle portion of flanks of Athymic nude mice. Mice were observed overtime for tumor formation. Mice implanted with the cell lines STS26T and NF90-8 developed tumors 4 and 12 weeks after cell implantation, respectively. MPNST xenografts are shown on Figure 2. Mice implanted with the remaining cells are currently under observation for tumor formation.

Task 3- Establishment of NF1 isogenic cells

A) Suppression of NF1 expression in MPNST cells. NF1 tumor suppressor loss is one of the most common genetic alterations in MPNST tumors. It is anticipated that finding a drug that shows specificity to cells lacking NF1 expression will represent a major step toward developing an efficacious therapy for MPSNT tumors. Therefore, we developed NF1 isogenic cells. These cells have the same genetic background and only differ on the NF1 gene expression. To that end, we selected the NF1 positive cell STS26T and proceeded to attain the constitutive knockdown of NF1 gene. Figure 3 shows the expression of NF1 RNA levels in the MPNST cells. To mimic NF1 expression loss we transduced lentiviral shRNA constructs specific for NF1 gene. The shRNA system selected was the Hairpin-pLKO.1 from the RNAi Consortium (TRC), available at the Genomics Resource core facility at JHU. We selected five independent NF1 shRNA clones: TRCN39713, TRCN39714, TRCN39715, TRCN39716 and TRCN39717. The NF1 hairpins are cloned into the lentiviral pLKO.1 vector. The non targeting GFP shRNA was used as a control. The viruses containing the shRNA constructs were transfected into STS26T cells using Lipofectamine 2000. Forty-eight hours later clones were selected with 2µg/ml of puromycin for 10 days. All transfections generated viable clones and were passaged in culture twice before testing the extent of the NF1 knockdown. Transfection of NF1 shRNA resulted in suppression of endogenous NF1 expression, as shown by the relative expression of NF1 RNA measured by RT-PCR (Figure 4). Next, the NF1 shRNA clones were tested for their proliferation rates. Cells were counted and 1,500cell/well were plated in 6 replicates in a 96-well. Alamar Blue reagent (Invitrogen) was added to the well and its fluorescence was read over a period of 120h in a Victor-3 plate reader (Perkin-Elmer). Suppression of NF1 (clone TRCN39716) was accompanied of increased cell proliferation, compared to the GFP shRNA control (Figure 5). Therefore, we selected the clone TRCN39716 for the subsequent drug screening. NF1shRNA and GFPshRNA cells were plated in 96-well plates (1500cells/well) and subjected to various concentrations of the inhibitors: Tandutinib, Sunitinib, Rapamycin, Gefitinib and Lapatinib. Control cells were plated with DMSO, used as vehicle for drug dilutions. Alamar Blue reagent was added to plates and its fluorescence was measured daily. The drugs half inhibitory concentration (IC_{50}) value was calculated at 72h after drug exposure. IC_{50} assays were analyzed by using GrapgPad Prism software. Table 2 and Figure 6 show the results of the initial IC_{50} assays. Using these NF1 isogenic cells we are currently focusing on the screen of a larger library, composed of FDA approved drugs.

B) NF1 ectopic expression in normal arachnoidal cells. The NF1 cDNA (clone 88:12) was kindly provided by Dr. F Hannan PhD (New York Medical College, Valhalla, NY). The NF1 plasmid construct was double-digested with Not-I and Sal-I and the NF1 insert was ligated into the linearized retroviral vector pBicep-CMV2 (Sigma). The resulting DNA construct was transformed and expanded in DH5-alpha cells. Empty vector pBicep-CMV2 and pBicep-CMV2-NF1 were transiently transfected into normal arachnoidal cells using Lipofectamine 2000. The level of NF1 expression was assessed by real time PCR. NF1 ectopic expression was increased >100 fold compared to the empty vector control (Figure 7). Next, we tested these cells for their ability to respond to drug treatments. We performed an IC_{50} assay as described above. A graphic plot with this IC_{50} assay resulted from the exposure to the drug Gefitinib is shown on Figure 8. We anticipate using these resources as a secondary drug screen for drugs that present specificity for cells with NF1 gene.

KEY RESEARCH ACCOMPLISHMENTS

1- We have developed 3 new MPNST cell lines from surgical specimens.
2- We have established an *in vivo* xenograft model in Athymic nude mice by implanting MPNST cells.
3- We developed NF1 isogenic cells appropriated for screening of large libraries of anti-neoplastic FDA approved drugs.

REPORTABLE OUTCOMES

We have successfully developed *in vitro* and *in vivo* MPNST models. These models are currently being used to attain our primary goal of finding tyrosine kinase inhibitors that effectively arrest the growth of MPNST cells.

CONCLUSION

The aim of our research is to identify and characterize an effective combination of tyrosine kinase inhibitors that show specificity to MPNST cells. In particular, we are interested in using NF1 isogenic MPNST cells. To that end, it is of importance the reagents developed during the first year of this project. We successfully developed three new MPNST cells and acquired four other established cell lines from outside collaborators. Moreover, we tested these cells for their capability to form tumor xenograft in nude mice. In addition, we developed a pair of NF1 isogenic cells, where the only difference is the NF1 gene expression. These cells are currently being employed to screen libraries of FDA approved drugs that are safe for human use. We anticipate that this strategy is very promising and will provide relevant information regarding the development of an alternative and efficacious therapy for NF1 patients.

So What? We have established new MPNST cell lines, drug screening assays and animal models crucial for drug screening and preclinical testing. We have entered the second phase of the research to identify which FDA approved drugs show the best activity against MPNST cells that have lost NF1. The goal of this work is to be gain supporting evidence so that a clinical trial can be planned to test existing drugs against MPNST with NF1 loss.

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SUPPORTING DATA

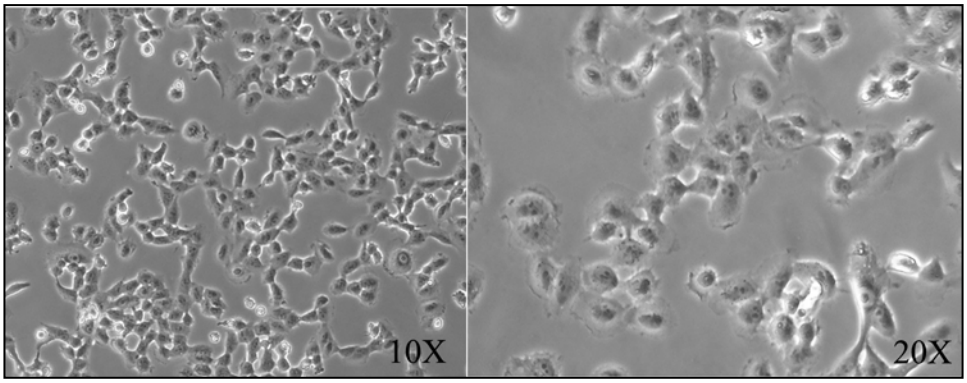

Figure 1 – Cell morphology characteristic of MPNST cells growing in culture. The 2010-013 cells growing in monolayer are observed.

Table 1 – MPNST cells established at Johns Hopkins and obtained from outside collaborators

MPNST Cell	Source
2010-013	Johns Hopkins Hospital
2011-005	Johns Hopkins Hospital
2011-007	Johns Hopkins Hospital
NF90.8	Michael Tainsky, Wayne University, Detroit, MI
ST8814	Michael Tainsky, Wayne University, Detroit, MI
STS26T	Steven Porcelli, Albert Einstein College of Medicine, Bronx, NY
T265-2C	Steven Porcelli, Albert Einstein College of Medicine, Bronx, NY

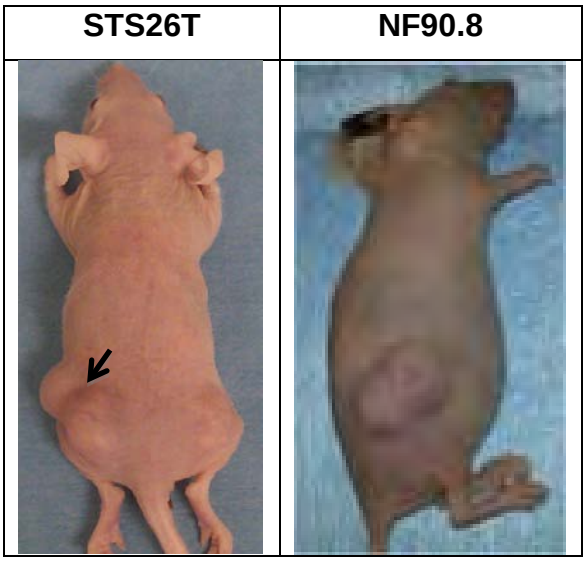

Figure 2 – Athymic nude mice showing flank xenografts of STS26T and NF90.8 MPNST cells.

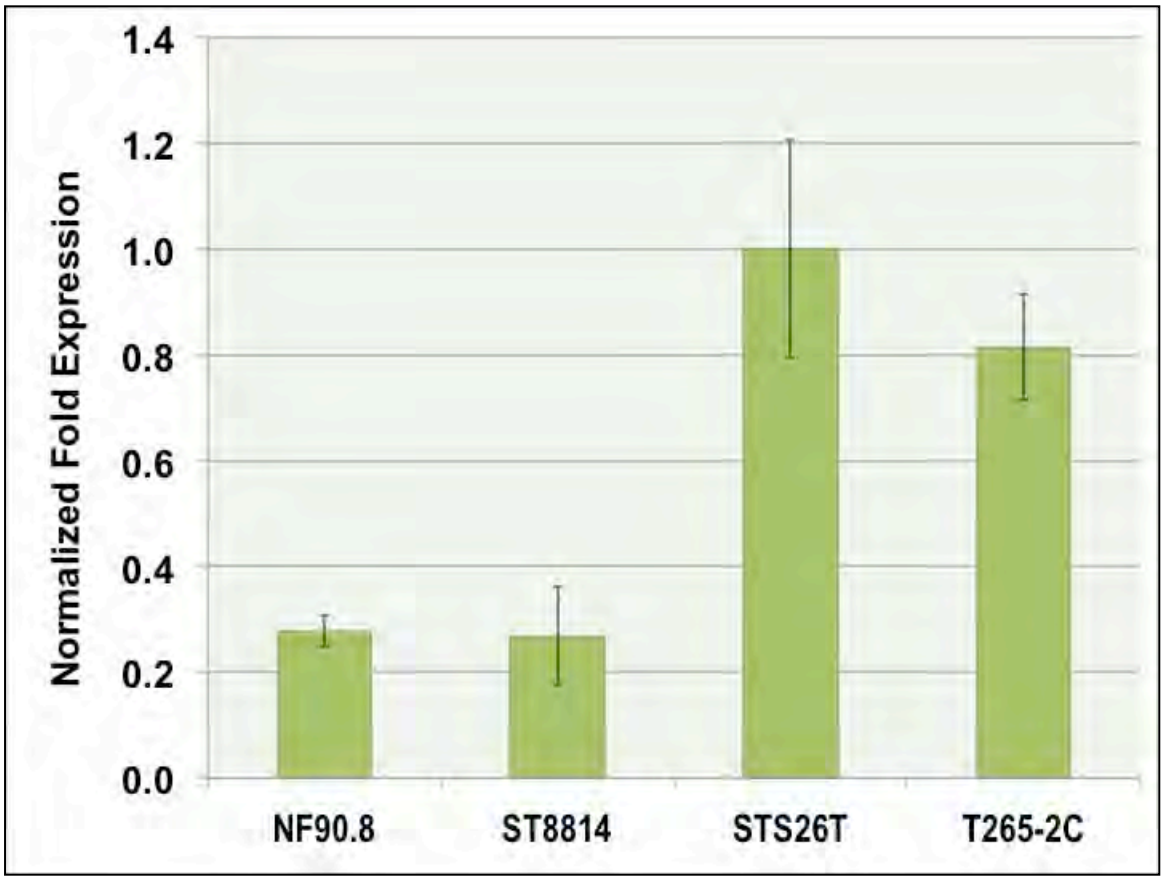

Figure 3 – Relative expression of NF1 gene in MPNST cells. The expression of NF1 gene was quantified by RT-PCR using TaqMan probe Hs01035108_m1 (Applied Biossystems), following manufacturer's instructions. NF1 relative expression was normalized to the expression of the house keeping transferrin receptor gene.

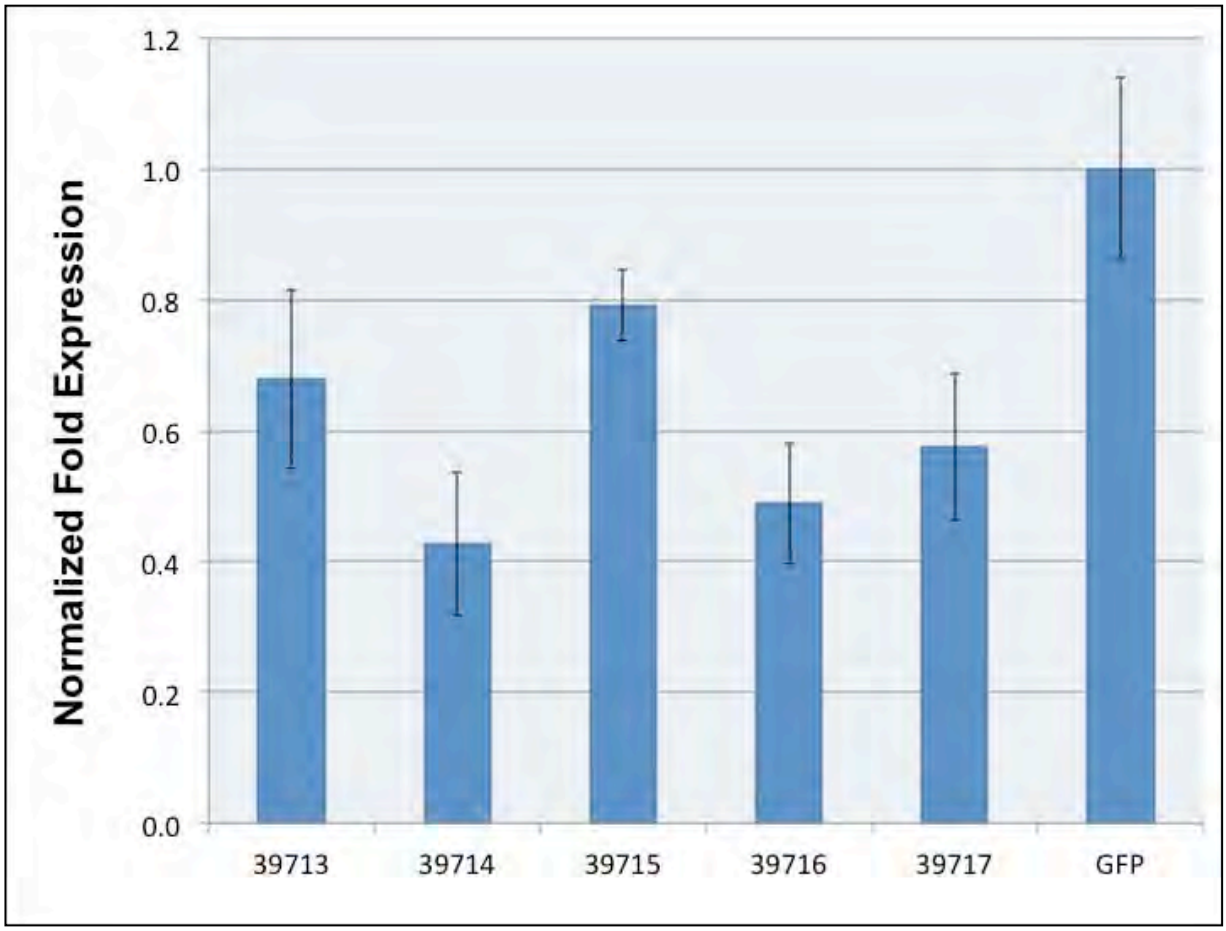

Figure 4 - Relative expression of NF1 gene in NF1 and GFP shRNA transfected cells. The expression of NF1 gene was quantified by RT-PCR using TaqMan probe Hs01035108_m. (Applied Biossystems), following manufacturer's instructions. NF1 relative expression was normalized to the expression of the house keeping transferrin receptor gene.

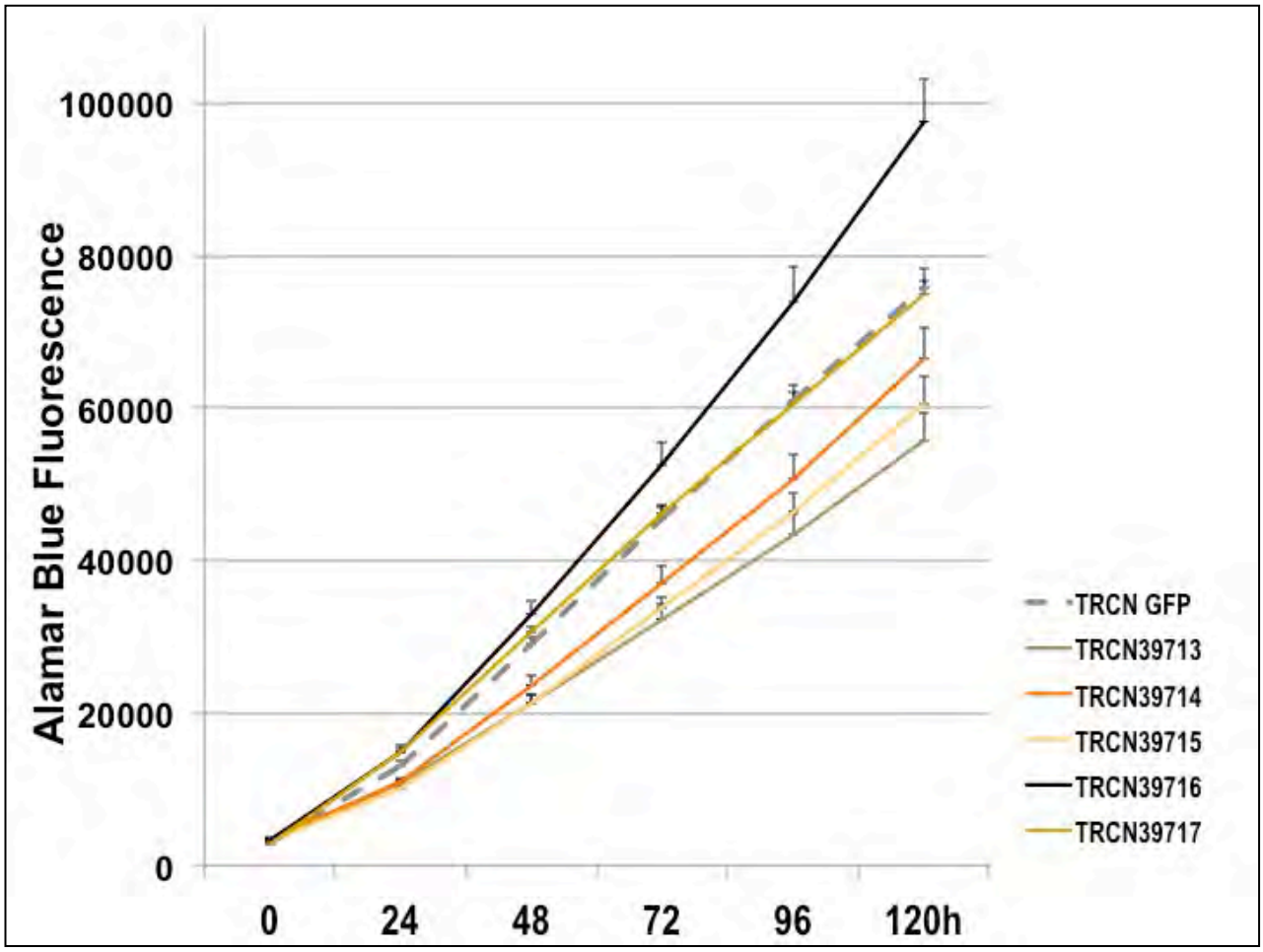

Figure 5 – NF1 knockdown promotes cell proliferation. Alamar Blue proliferation assay of STS26T cells transduced with NF1 and GFP shRNA lentiviral constructs.

Table 2 – IC_{50} values for MPNST cells with constitutive NF1 knockdown compared to the control (GFP shRNA).

	GFP shRNA	NF1 shRNA
Tandutinib	120uM/$R^2$0.93	52/$R^2$0.90
Lapatinib	7.5uM/$R^2$0.98	6uM/$R^2$0.91
Gefitinib	20uM/$R^2$0.93	16uM/$R^2$0.99
Rapamycin	13uM/$R^2$0.96	13/$R^2$0.91
Sunitinib	11.5uM/$R^2$0.98	16uM/$R^2$0.93

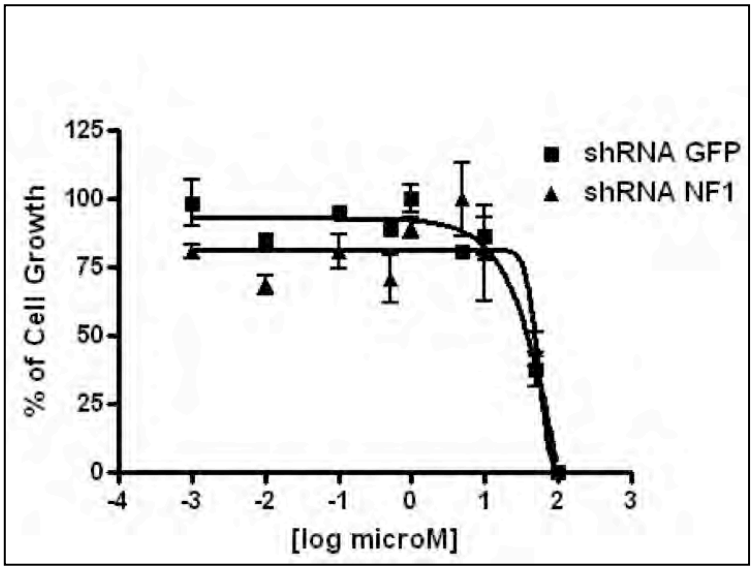

Figure 6 – IC50 assay of MPNST cells. Cells transduced with either non targeting GFP shRNA or NF1 shRNA and subsequently subjected to treatment of the kinase inhibitor Tandutinib in various concentrations.

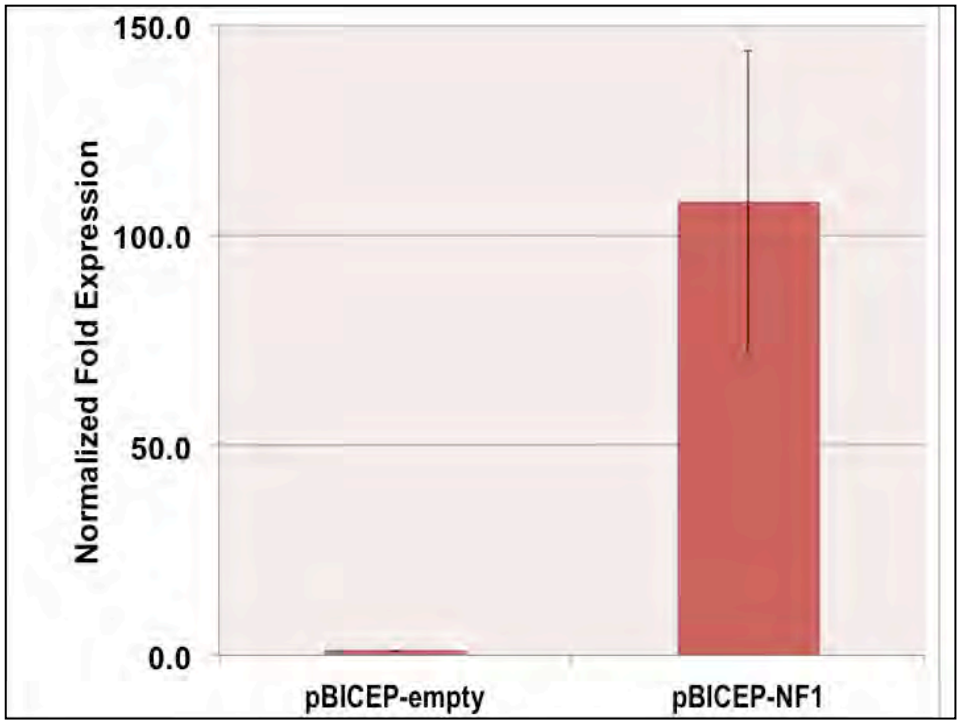

Figure 7 - Relative expression of NF1 gene in normal arachnoidal cells transfected with either the NF1 construct or empty vector. The expression of NF1 gene was quantified by RT-PCR using TaqMan probe Hs01035108_m. (Applied Biossystems), following manufacturer's instructions. NF1 relative expression was normalized to the expression of the house keeping transferrin receptor gene.

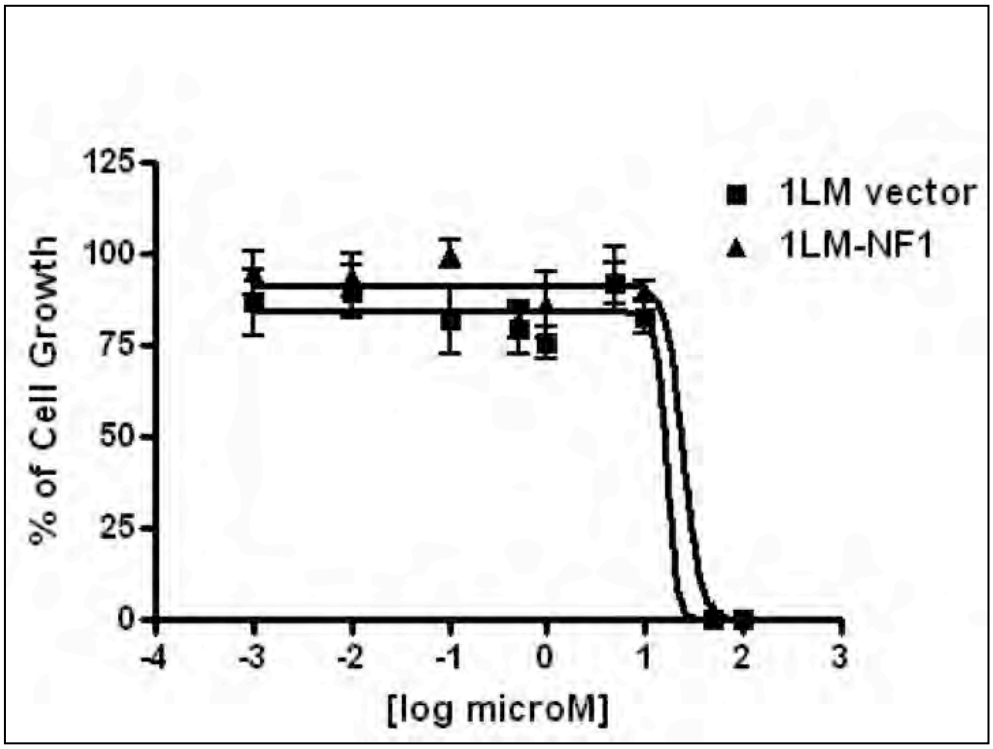

Figure 8 – IC50 assay of normal arachnoidal cells. Cells transfected with either NF1 expression construct or the empty vector and subsequently subjected to treatment of the kinase inhibitor Gefitinib in various concentrations.